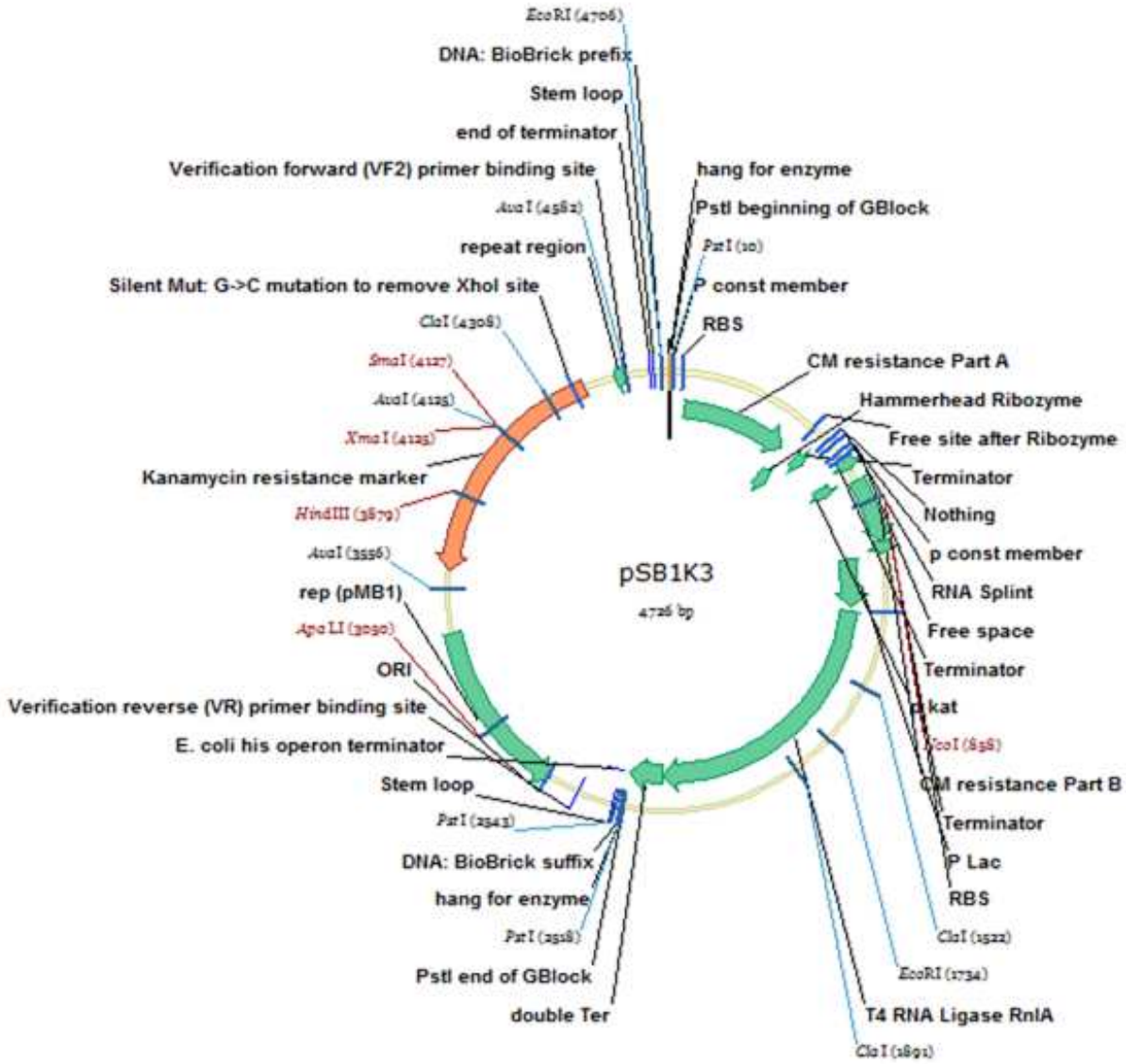
# CM Split




PART 1: (870)

ATGCCTGCAGTTTACAGCTAGCTCAGTCCTAGGTATTATGCTAGCATTAAAGAGGAGAAAATGGAGAAAAAAATCAC
TGGATATACCACCGTTGATATATCCCAATGGCATCGTAAAGAACATTTTGAGGCATTTCAGTCAGTTGCTCAATGTACC
TATAACCAGACCGTTCAGCTGGATATTACGGCCTTTTTAAAGACCGTAAAGAAAAATAAGCACAAGTTTTATCCGGCC
TTTATTCACATTCTTGCCCGCCTGATGAATGCTCATCCGGAATTTCGTATGGCAATGAAAGACGGTGAGCTGGTGATA
TGGGATAGTGTTCACCCTTGTTACACCGTTTTCCATGAGCAAACTGAAACGTTTTCATCGCTCTGGAGTGAATACCACG
ACGATTTCCGGCAGTTTCTACACATATATTCGCAAGATGTGGCGTGTTACGGTGAAAACCTGGCCTATTTCCCTAAAG
GGTTTATTGAGAATATGTTTTTCGTAACCGGATGTGTTTCCGGTCTGATGAGTCCGTGAGGACGAAACGAAAAAATGC
CAGTTATGGAACATAGACTCACACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTATAAGTTACCGTAATGCAATGC
ATTTACAGCTAGCTCAGTCCTAGGTATTATGCTAGCGGGATTGGCTAAAACATATTATACGAACTGTCACACTGGCTC
ACCTTCGGGTGGGCCTTTCTGCGTTTATACATTATTGCAATTAATAAACAACTAACGGACAATTCTACCTAACATCAGC
CAATCCCTGGGTGAGTTTCACCAGTTTTGATTTAAACGTGGCCAATATGGACAACTTCTTCGCCCCCGTTTTCACCATG
GGCAAATAT

PART : (1673)

GTTTTCACCATGGGCAAATATTATACGCAAGGCGACAAGGTGCTGATGCCGCTGGCGATTCAGGTTCATCATGCCGTT
TGTGATGGCTTCCATGTCGGCAGAATGCTTAATGAATTACAACAGTACTGCGATGAGTGGCAGGGCGGGGCGTAATC
ACACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTATACAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCA
TTAATGCAGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAACGCAATTAATGTGAGTTAGCTCA
CTCATTAGGCACCCCAGGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGATAACAATTTCA
CACAATTAAAGAGGAGAAAATGCAAGAACTTTTTAACAATTTAATGGAACTATGTAAGGATTCGCAGCGTAAGTTTTT
TTACTCAGATGATGTAAGTGCATCTGGAAGAACTTACAGAATTTTCTCATATAATTATGCATCTTATTCTGATTGGTTAC
TTCCAGATGCACTAGAATGTCGTGGAATTATGTTTGAAATGGATGGAGAAAAACCAGTAAGAATTGCTTCTCGTCCTA
TGGAAAAGTTTTTTAACTTGAATGAAAATCCGTTCACGATGAATATCGATTTAAACGATGTTGATTATATTCTAACAAA
AGAAGACGGGTCTTTGGTATCAACTTATTTAGACGGTGATGAAATTCTGTTCAAATCAAAGGGTTCAATCAAATCTGA
GCAGGCTTTAATGGCTAATGGAATTTTGATGAATATTAATCACCATCGGTTGCGTGATAGACTTAAAGAATTAGCTGA
AGATGGATTTACTGCTAACTTCGAATTCGTTGCCCCGACGAATAGAATCGTTCTTGCTTATCAAGAGATGAAAATTATT
TTACTGAATGTTCGTGAAAACGAAACGGGTGAATACATTTCATACGATGATATTTATAAAGATGCTACTCTTCGTCCGT
ATCTAGTTGAACGATACGAAATCGATAGCCCTAAATGGATAGAAGAAGCTAAAAATGCAGAAAACATCGAAGGCTAT
GTTGCTGTGATGAAAGATGGTTCTCATTTTAAAATTAAGTCTGACTGGTACGTGTCTCTTCATAGTACAAAAAGTTCAT
TAGATAATCCAGAAAAATTGTTTAAGACTATTATTGATGGTGCATCAGATGATCTTAAAGCAATGTATGCTGACGATG
AATATTCATACAGAAAAATTGAAGCATTTGAAACGACTTATCTGAAGTACTTAGACCGAGCTCTGTTTTTAGTTCTTGA
CTGTCATAATAAGCATTGCGGTAAGGATAGAAAGACTTATGCAATGGAAGCACAAGGTGTTGCTAAAGGTGCTGGAA
TGGATCACCTGTTCGGTATCATCATGAGCTTATACCAGGGGTACGATAGTCAAGAAAAGGTCATGTGTGAAATCGAA
CAGAATTTTTTGAAAAATTATAAAAAATTTATCCCAGAAGGATACTAACCAGGCATCAAATAAAACGAAAGGCTCAGT
CGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTCTCTACTAGAGTCACACTGGCTCACCTTCGGG
TGGGCCTTTCTGCGTTTATACTGCAGAGCT

The first step is to amplify the two gBlocks:

# The PCR reaction:

**21/09/2014**

**Reaction mix:**
**GB Part 1 (870):**

| Competent | Volume (µl) |
|---|---|
| Phusion reaction buffer (X10) | 10 |
| dNTP's (10 mM) | 1 |
| GB1 Forward | 2.5 |
| RNA GB1  Reverse | 2.5 |
| RNA GB1 (20 ng/µl) | 0.5 |
| Phusion hot start II | 0.5 |
| DMSO | 1.5 |
| UPW | 31.5 |
| Total | 50 |

**PCR program**

| Stage | Temperature (°C) | Time |
|---|---|---|
| Initial denaturation | 98 | 30 sec |
| 35 cycles | 98<br>67<br>72 | 10 sec<br>30 sec<br>30 sec |
| Final extension | 72 | 10 min |
| Hold | 4 | |

**01/10/2014**

**GB Part 2 (1673):**

| Competent | Volume (µl) |
|---|---|
| Phusion reaction buffer (X10) | 10 |
| dNTP's (10 mM) | 1 |
| RNA GB2 Forward | 2.5 |
| RNA GB2 Reverse | 2.5 |
| GB2 (20 ng/µl) | 0.5 |
| Phusion hot start II | 0.5 |
| DMSO | 1.5 |
| UPW | 31.5 |
| Total | 50 |

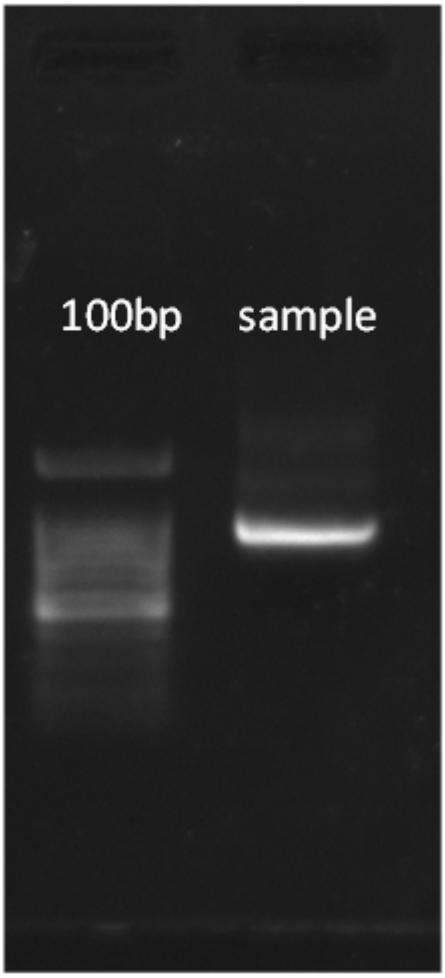
**PCR program**

| Stage | Temperature (°C) | Time |
|---|---|---|
| Initial denaturation | 98 | 30 sec |
| 35 cycles | 98<br>64<br>72 | 10 sec<br>30 sec<br>1 min |
| Final extension | 72 | 10 min |
| Hold | 4 | |

After the PCR we ran the product in gel and expect the following bands:
For the reaction of GB1: 870 bp
For the reaction of GB2: 1673 bp

After that we cleaned the PCR product

The PCR succeeded: expected band was 870bp.




**04/10/2014**

The next step was:

## Restriction enzymes:

NcoI and PstI both are HF so the buffer is CUTSMART
Cut of the 2 Gblocks (after PCR)

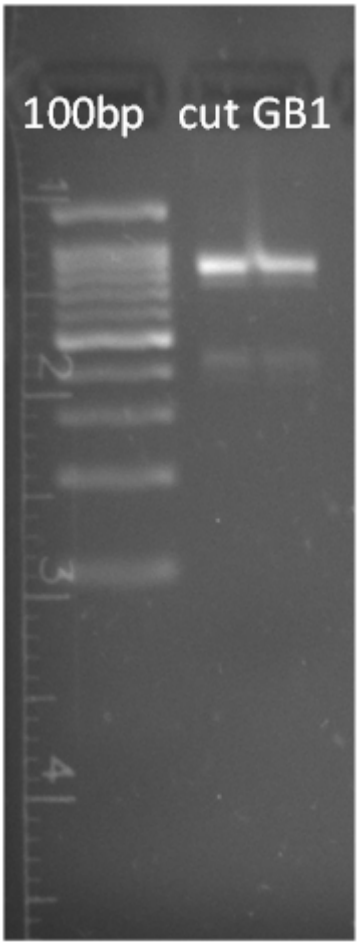
**The reaction:**

**For GB1:**

| $\mu l$ | |
|---|---|
| 26 | DNA (77ng/ul) |
| 0.5 | PstI HF |
| 0.5 | NcoI HF |
| 3 | Cutsmart |
| 0 | mbw |
| 30 | total |

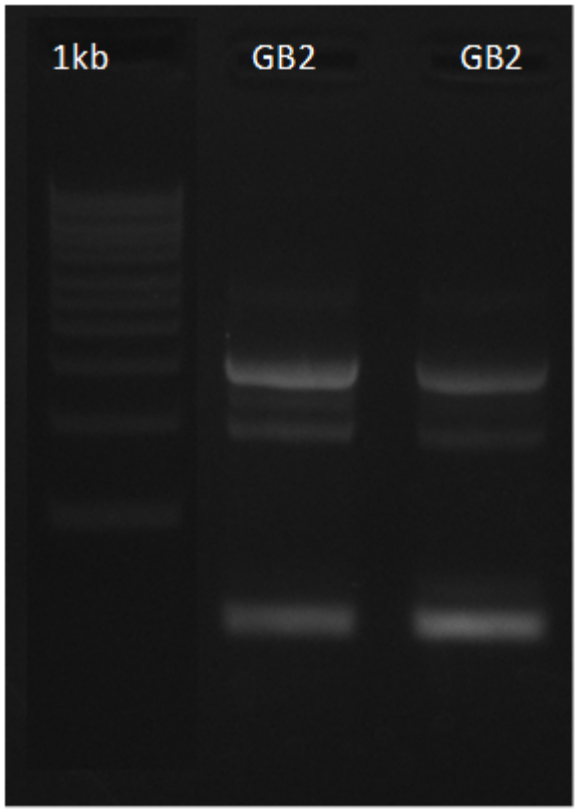
**The gel of the cut GB-1:**

As expected although it is not seen differently.




**For GB2:**

| $\mu l$ | |
|---|---|
| 53 | DNA (33.5ng/ul) |
| 0.5 | PstI HF |
| 0.5 | NcoI HF |
| 6 | Cutsmart |
| 0 | mbw |
| 60 | total |




The PCR succeeded expected is 1673bp.
A clean from gel was done and the concentration is 17.1 ng/ul.

**For pSB1k3:**

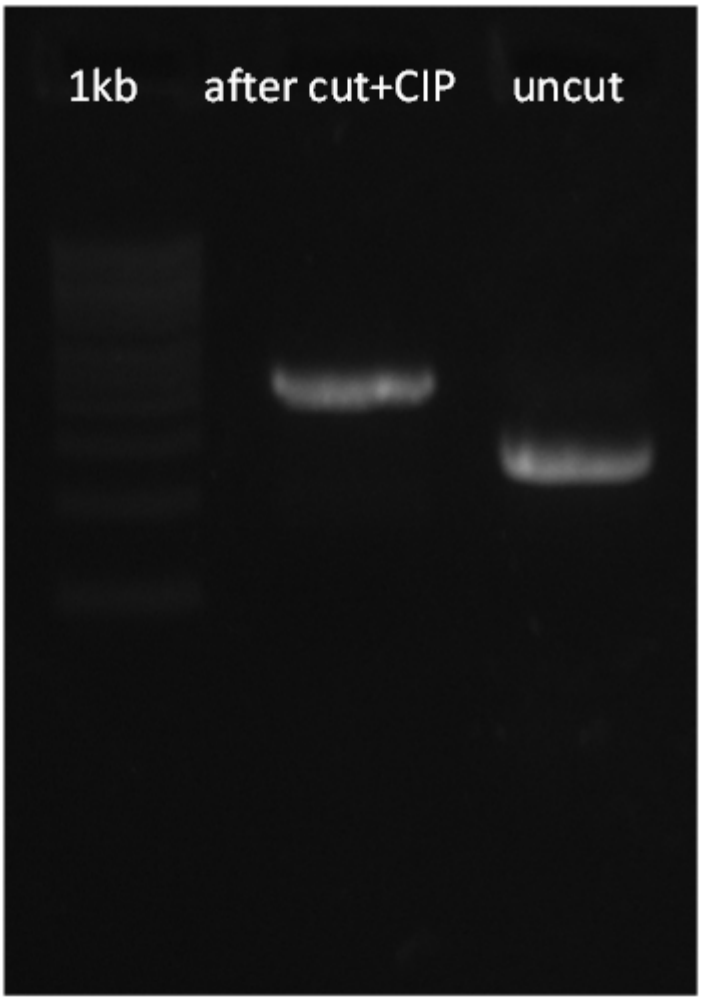
| $\mu l$ | |
|---|---|
| 35.5 | DNA (84ng/ul) |
| 0.5 | PstI HF |
| 4 | Cutsmart |
| 0 | mbw |
| 40 | total |

And the plasmid pSB1K3 was cut only with PstI (the miniprep is in the box of Gate 1)

**The reaction:**

| $\mu l$ | |
|---|---|
| 35 | DNA (pSB1k3) |
| 1 | CIP (NEB) |
| 4 | Buffer 3 |
| 0 | mbw |
| 40 | total |

60 min at 37°C




As it can be seen from the gel, the cut was succeeded.

After the digestion we ran a gel and saw the right bands:
For the reaction of GB1:~ 850 bp
For the reaction of GB2:~ 1650 bp
For the plasmid expect to get: 2204 bp

After that cleaned the product (clean from PCR)
For the plasmid did CIP and then a second clean (clean from PCR)

**05/10/2014**

## Ligation

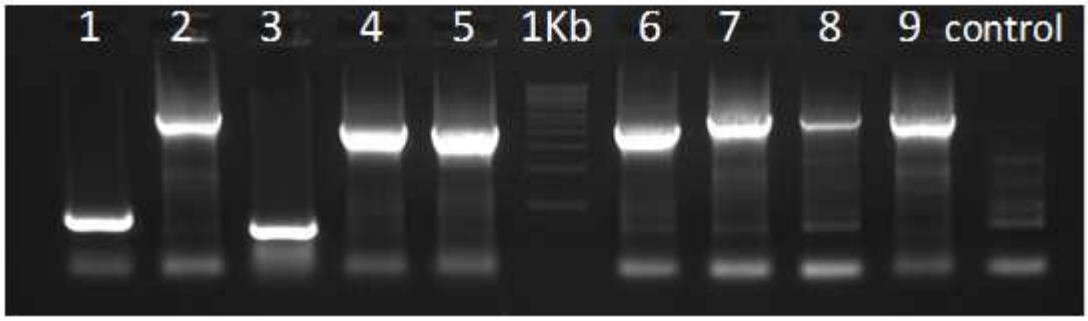
The next step is ligation of the three parts-was done according to the concentration after the cut and clean.
Ligation:

**The reaction:**

| | |
|---|---|
| GB 1 (35.5 ng/ul) | 60ng=1.7ul |
| GB 2 (17.1 ng/ul) | 115ng=6.8ul |
| plasmid (PSB1K3)(46 ng/ul) | 50ng=1.1ul |
| mbw | 7.4 |
| DNA Ligase buffer | 2 |
| T4 DNA Ligase | 1 |
| total | 20 |

O/N at 16°C

Finally did transformation and colony PCR.
After colony PCR, colony 8 was chosen. (expected band 2800bp)




In order to do the experiment the plasmid was minipreped from colony 8 (520 ng/ul)

**07/10/2014**

# Transformation

After the miniprep the plasmid transformed to DH5Alphaz1 (has many copies of the lac repressor so it is easier to see the CM resistance only after inducing with IPTG to express the T4 RNA Ligase).

**13/10/2014**

# Experiment

After transformation the bacteria was grown O/N in LB+Kan and after that induced for 5 hr (usually 3 but here we express few proteins the CM resistance will be at the end, after the T4 RNA Ligase is expressed). After that the bacteria was plate on agar with Kan+CM+IPTG and grown on LB+Kan+CM+IPTG.

<u>The experiment:</u>

Grow the bacteria, DH5Alphaz1, with the plasmid pSB1K3+GB1+GB2 O/N (5ml starter).

2x Transfer from the starter 1ml to 100ml LB with kan and culture to 0.6 O.D.

Add IPTG to one of them (1mM) and culture 4hr (usually it is for 3hr but here there is an expression of few proteins in serial; first the T4 RNA Ligase and then the CM Resistance. 3hr as normal+1hr as the time for recovery).

Spread on plate containing CM+Kan/CM+Kan+IPTG (depends on the culture) (100ul+rest). (4 plates in total).

Transfers 1ml to 100ml LB, do it for both of the cultures (both LB with Kan+CM and only one with IPTG).

Incubate the plates and the LB O/N.

For the plates: count colonies.

For the LB: read O.D.

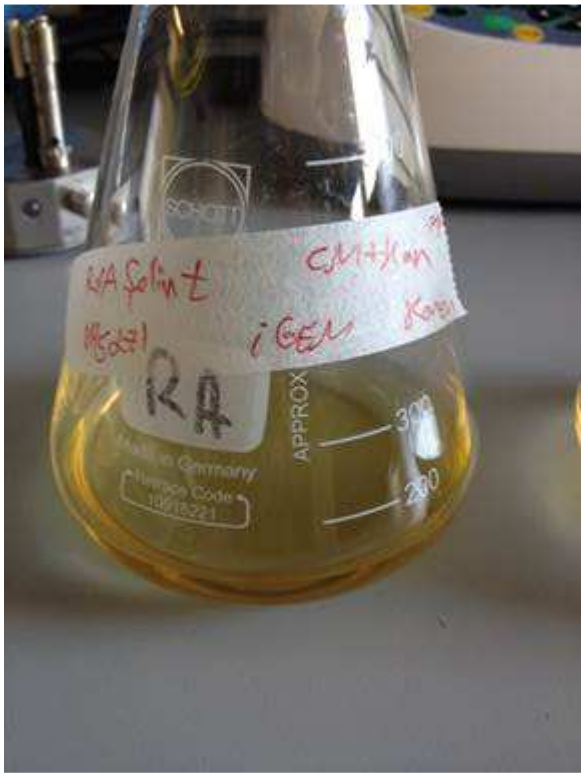
Expect for higher O.D. and more colonies while inducing with IPTG.

Results:

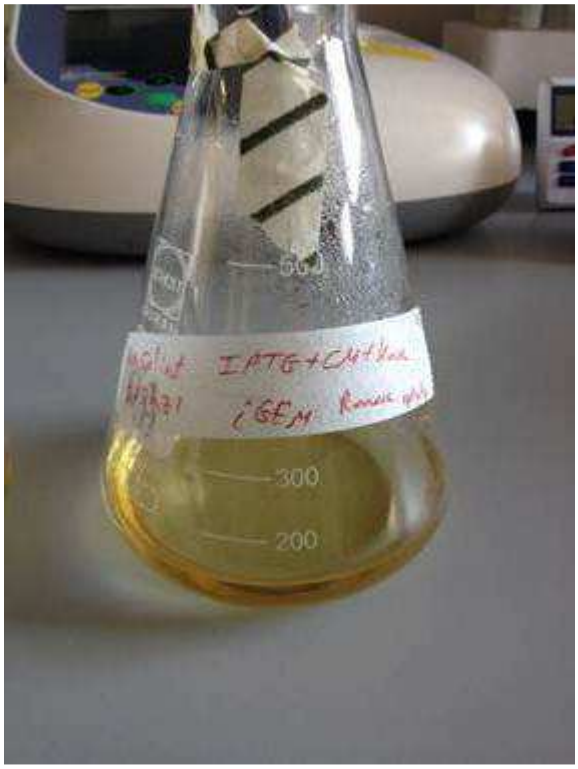
Although it was expected to be seen differently than the control, unfortunately both of the cultures, control and experiment, were negative.

The O.D. was as follows (after the O/N growth):

| | $O.D._{600nm}$ |
|---|---|
| Experiment (LB+CM+Kan+IPTG) | 0.14 |
| Control (LB+CM+Kan) | 0.08 |



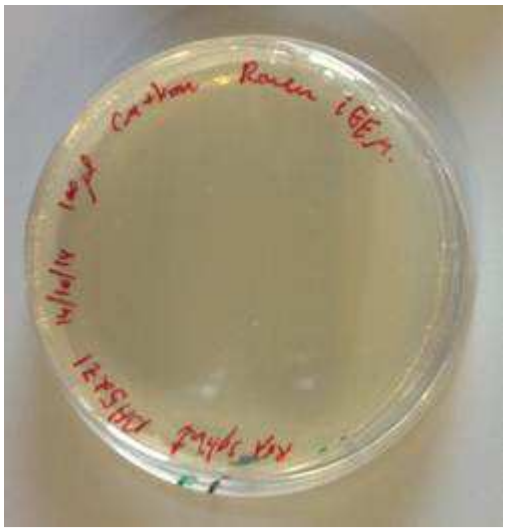

Pic 1: Control LB after O/N incubation



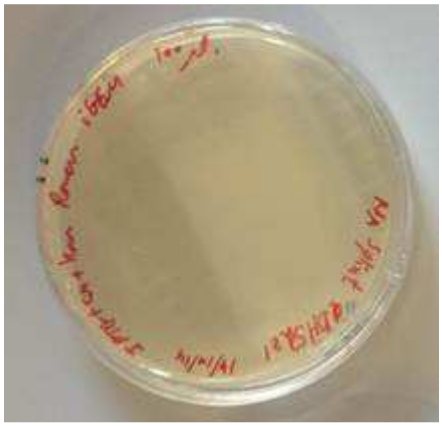

Pic 2: Experiment LB after O/N incubation (with IPTG)

-

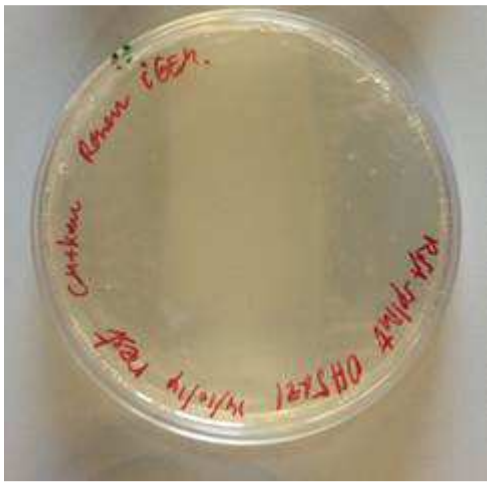
As for the plates, all of the 4 plates (2 cultures each 100ul and rest) were blank; no colonies grew after O/N incubation.



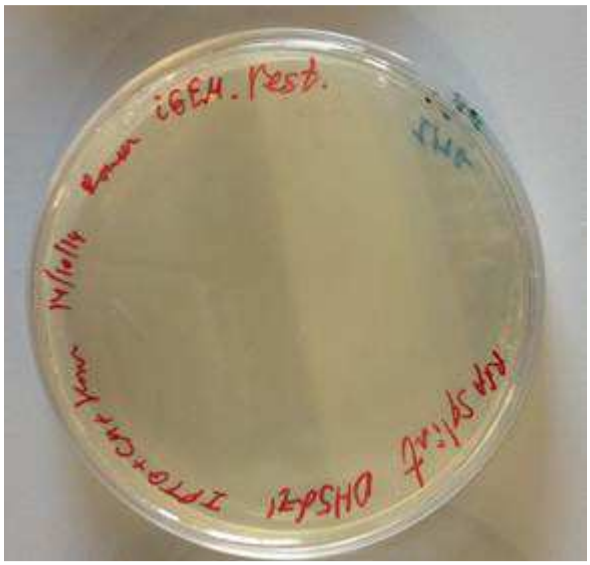
Pic 3: Control plate (CM+Kan) 100ul



Pic 4: Experiment plate (CM+Kan+IPTG) 100ul



Pic 5: Control plate (CM+Kan) rest



Pic 6: Experiment plate (CM+Kan+IPTG) rest

# Future work

For future plan we need to check different designs of the splint, maybe different length.
We can use a verity of splints and check them.

Another thing we can try (and probably do so before the Jamboree) is to use a variety of concentrations of CM and look for resistance in lower concentrations. Also we plan on titrating the CM after the incubation with our strain and compare it to a non CM resistance strain, to see if there is any degradation of the CM by the bacteria.